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Laboratory Protocol

Protocol number: 2
Protocol description: qPCR for DNA samples of unknown quantity
Original reference: Morin et al. (2001) Molecular Ecology 10:1835-1844
Original entry: Steve Smith October 2001
Last updated: October 2001
Updated by: Steve Smith

Required materials:
starred (*) reagents are described in the 'standard laboratory solutions' document)

1. CMYC taqman assay reagents
   - forward primer = cMYC_E3_F1U1 (AGAGGAGGAACGAGCT)
   - reverse primer = cMYC_E3_R1U1 (GGGCCTTTTCATTGTTTTCCA
   - probe = cMYC_E3_TMV (TGCCCTGCGTGACCAGATCC
   - Eurogentec Taqman reagents (qPCR core kit/RT-QP73-05):
     - § 10 x master mix
     - § $MgCl_2$ (25 mM)
     - § dUTP's (2.5 mM)
     - § Hotstar Gold Taq polymerase (5 U/µl)
   - Bovine Serum Albumin (BSA) (20 mg/ml)
   - Uracil – n – glycosylase (UNG) (1 U/µl)
2. Perkin Elmer (PE) DNA standard dilution plate for the 7700 sequence detector prepared following the qPCR Dilution Series protocol (Protocol #1)
3. PE optical 96-well PCR plates (N801-0560)
4. PE optical strip caps for sealing the plate. (4323032)

Required equipment:

1. 7700 Sequence Detector ("Taqman" instrument)

Protocol:

1. Design your experiment and enter the sample names into the Datasheet worksheet of the Experiment Workbook (see QPCR excel workbook and figure 1 below). For each set of samples to be quantified in one experiment, triplicate standards need to be run, along with several no-template controls and the samples. If more samples are to be run than can fit on a 96-well plate, they can be amplified sequentially in the QPCR instrument (using one PCR master mix) and analyzed with the same standard.

(standards)

WELL #	Sample ID:	real-time collection data DNA conc'n (pg)	Ct	log (pg)
A1	PE std, 60 ng	60000.00		4.78
A2	PE std, 30 ng	30000.00		4.48
A3	PE std, 15 ng	15000.00		4.18
A4	PE std, 7.2 ng	7200.00		3.86
A5	PE std, 3.0 ng	3000.00		3.48

(unknowns)

E1	unknown 1			#NUM!
E2	unknown 2			#NUM!
E3	unknown 3			#NUM!
E4	unknown 4			#NUM!
E5	unknown 5			#NUM!
E6	unknown 6			#NUM!
E7	unknown 7			#NUM!
E8	unknown 8			#NUM!
E9	unknown 9			#NUM!

2. In the no-DNA hood prepare a mastermix for the cMYC quantitative PCR assay using the following conditions (adjusted for the number of samples). You will need enough for 36 tubes of standards and 2 replicates of each of your DNA samples (plus ≥3 no template controls (NTCs)).

EXPERIMENT NAME:

INVESTIGATOR:

Assay Name	cMYC #1
probe	cMYC_E3_TMV
Forward Primer	cMYC_E3_F1U1
Reverse Primer	cMYC_E3_R1U1

					# TUBES TO SETUP:		**48**
P		**PERCENT PIPETTE EXCESS:**		10			
C	**MASTER MIX:**	**STOCK CONC.**	**FINAL CONC.**	**UNITS**	**1X VOLS.**		**USE (mL)**
R	10X PCR master mix	2	1	X	2.0		**105.6**
	BSA	100	0	%	0.8		**42.2**
C	25 mM MgCl2	25	0	mM	3.6		**190.1**
A	1:1:1:2 d(A,C,G,U)TPs*	2500	0	mM*	2.0		**105.6**
L	Taq Gold Polymerase (2.5 U/µL)	2.5	0	U/µL	0.1		**5.3**
C	fwd primer	20000	300	nM	0.3		**15.8**
U	rev primer	20000	300	nM	0.3		**15.8**
L	Probe	20000	200	nM	0.2		**10.6**
A	AmpErase (1U/uL)	1	0	U/µL	0.1		**5.3**
T	DI water		-	-	5.6		**295.7**
O	**[dUTP] = 2X[dNTP] in NTP mix*		MASTER MIX total		15.0		**792.0**
R	**Template DNA or dH2O**				5.0		
	REACTION TOTAL (ALL COMPONENTS):			**1X VOL:**	20.0		**792.0**

	temp	time
step 1	50°	2 Min
step 2	95°	10 Min
step 3	95°	15 Sec
step 4	59°	30 Sec
step 5	goto step 3, 49 times	
step 6	20°	1 Min
step 7	End	
step 8		
step 9		

3 In the DNA hood, add 5 µl of sample DNA to wells in rows D to H of a pre-prepared PE Standard plate (see protocol # 1 on preparation of PE Standard plates). Ensure that you have duplicates of each of your samples and to include at least 3 NTC's. Samples that cannot go on the first plate can be put into additional plates or tube-strips (optical quality).

4 Use the multichannel pipette to add 15 µl of master mix into each well. Ensure that you change tips after each row to avoid cross-contamination. Cap the tubes with optical 8-strip lids.

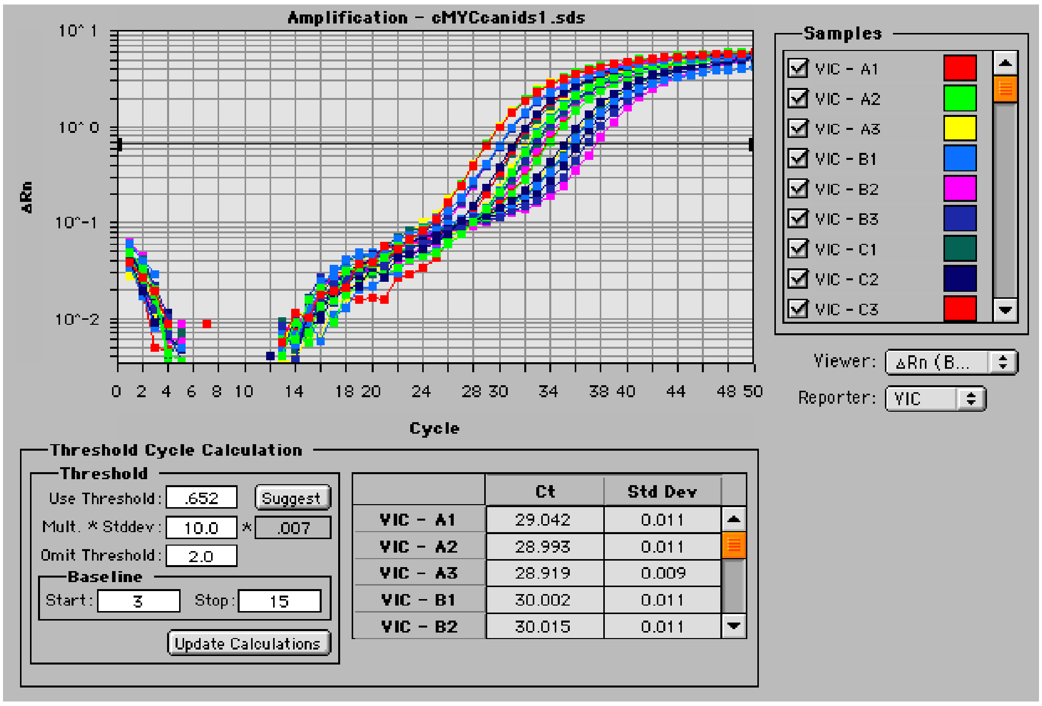
Starting the QPCR run

1. Open a blank PEstd.sds template from the cMYC Taqman folder. This template is already set up with the PE standards in triplicate. Adjust the number of unknowns to reflect the number of samples in your plate. Highlight the empty wells and use the drop-down **Sample Type Menu** to change their status to “not in use”.
2. Check to see which reporter dye is on your taqman probe (usually VIC) and ensure that template is showing that dye layer for your assay set up.
3. Click on the box marked **Thermal Cycler Conditions** to make sure the proper conditions are listed. These should read as follows:
   Step 1: 50°C, 2 min
   Step 2: 95°C, 10 min
   Step 3: 95°C, 15 sec
   Step 4: 59°C, 30 sec
   Go To Step 3, 49 times
   Step 5: 20°C, 1 min
   Step 6: End

4. Click on **Show Analysis**.
5. Load your sample tray into the taqman machine ensuring that well A1 is in the top left corner, and close and screw down the lid.
6. Ensure that Appletalk is disconnected and then click **Run** on the Show Analysis screen. After the cycles have begun, the time to completion will be shown (typically 1 hour and 58 minutes).

Analyzing Data after a Taqman Run

1. Immediately use the “save as” option from the **File** menu to save your run to an appropriate folder.
2. Pull down the **Analysis** menu and select **Analyze.**
3. When you see the amplification plot window, do the following:
   Look at the figures in the **Baseline** boxes. Make sure the largest number is at least 3 cycles PRIOR to the beginning of the exponential amplification on the plot window. If it looks like it may be close, double click on the “ΔRn” (Y axis) and select “linear plot”, then check to see at what cycle the fluorescence values begin to rise above the baseline. If you need to change the maximum “baseline” cycle number, change it in the **Baseline** box, then change the plot back to “logarithmic”
   Establish the **Use Threshold** by clicking and holding on the black horizontal bar on the amplification plot. Move it to a point ABOVE the baseline and well WITHIN the area of exponential amplification. NOTE: If you ran your standards on a previous plate, record the value of the Use Threshold from the standard plate, and enter this number in the appropriate box at the lower left hand of the screen for each subsequent set of samples.
   If you make any changes to the values in the box at the lower left, click on **Update Calculations.**



4. Pull down the **File** menu, select **Export** and then choose **Results**.
5. When prompted, save the exported file as **<Filename>.results.** Make sure that **Export All Wells** is selected and then click on **Export** to complete the process.
6. Open your **<Filename>.results** file and copy the entire table .
7. Open your Excel **Experiment Workbook** in which you entered all the sample names. Click on the tab labeled **7700 Results** at the bottom of the sheet and paste the results table here (be sure to start at the first cell (A1).

	A	B	C	D	E	F	G	H	I	J	K	L
1	Well	Reporter	Type	Baseline StdDev	deltaRn	Ct	Quantity	Replicate	Qty Mean	Qty StdDev	IPC	Sample Name
2	1	VIC	STND	9.99E-03	5.11E+00	23.25	6.00E+04	1	1.07E+04	1.88E+04	+	PE Std
3	2	VIC	STND	1.18E-02	5.04E+00	24.3	3.00E+04	1	1.07E+04	1.88E+04	+	PE Std
4	3	VIC	STND	1.28E-02	4.90E+00	25.18	1.50E+04	1	1.07E+04	1.88E+04	+	PE Std
5	4	VIC	STND	9.08E-03	4.88E+00	26.51	7.20E+03	1	1.07E+04	1.88E+04	+	PE Std
6	5	VIC	STND	1.03E-02	4.69E+00	27.48	3.00E+03	1	1.07E+04	1.88E+04	+	PE Std
7	6	VIC	STND	7.42E-03	4.61E+00	28.67	1.50E+03	1	1.07E+04	1.88E+04	+	PE Std 1:10
8	7	VIC	STND	1.23E-02	4.53E+00	29.94	7.20E+02	1	1.07E+04	1.88E+04	+	PE Std 1:10
9	8	VIC	STND	9.60E-03	4.50E+00	31.14	3.00E+02	1	1.07E+04	1.88E+04	+	PE Std 1:10
10	9	VIC	STND	9.25E-03	4.39E+00	32.32	1.50E+02	1	1.07E+04	1.88E+04	+	PE Std 1:100

8. On the menu at the bottom of your **Experiment Workbook**, click on the **Standard Curve** worksheet tab to view an excel graph of the standard curve and the points used to construct the graph.
9. If the correlation coefficient (R^2) of the curve is not >0.98, check the graph for outlier points which may represent anomalous amplifications or failed reactions. Delete these outliers from the data table to the left of the graph to improve the R^2 value. As the curve is made up from 3 sets of identical standards, the removal of 1 or2 outliers will not unduly bias the plot because there are still 2 homologous points left from that exact same standard DNA.
10. Record the values for the slope, y-intercept and R^2 from the equation of the line for the standard curve.
11. On the menu at the bottom of your **Experiment Workbook**, click on the **Datasheet** tab. On the **Datasheet** fill in the three yellow boxes:

    Slope
    y-int
    R2

    with the values recorded from the standard curve.

	STANDARD CURVE	Slope	-3.4381		
		Y-intercept	39.805		
		R^2	0.9967		
WELL		real-time collection data			
#	Sample ID:	DNA conc'n (pg)	Ct	log (pg)	Lambda DNA Y/N
A1	PE std, 60 ng	60000.00	23.25	4.78	Y
A2	PE std, 30 ng	30000.00	24.30	4.48	Y
A3	PE std, 15 ng	15000.00	25.18	4.18	Y
A4	PE std, 7.2 ng	7200.00	26.51	3.86	Y
A5	PE std, 3.0 ng	3000.00	27.48	3.48	Y
A6	PE std 1:10, 1.5 ng	1500.00	28.67	3.18	Y
A7	PE std 1:10, 0.72 ng	720.00	29.94	2.86	Y
[illegible]	PE std 1:10, 0.3 ng	300.00	31.14	2.48	Y

		PE standard curve		
	UNKNOWNS	Slope	-3.4381	
		Y-intercept	39.805	
		R^2	0.9967	
WELL		real-time collection data		
#	Sample ID:	Ct	DNA template (pg)	DNA Template (ng)
A1	PE std, 60 ng	23.25	65,337	65.34
A2	PE std, 30 ng	24.30	32,341	32.34
A3	PE std, 15 ng	25.18	17,939	17.94
A4	PE std, 7.2 ng	26.51	7,361	7.36
A5	PE std, 3.0 ng	27.48	3,844	3.84
A6	PE std 1:10, 1.5 ng	28.67	1,733	1.73
A7	PE std 1:10, 0.72 ng	29.94	740	0.74
[illegible]	PE std 1:10, 0.3 ng	31.14	331	0.33

12. As soon as you have entered the above values, the DNA quantities for your unknown samples will be calculated automatically in the data sheet, columns J & K (pg and ng). (calculated from the equation: DNA amount = $10^{((Ct - Yint)/slope)}$).
13. **SAVE YOUR RESULTS.** Print out hard copies of the standard curve and datasheet for your lab notebook.